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Suppression of Intestinal Polyposis in $Apc^{\Delta716}$ Knockout Mice by Inhibition of Cyclooxygenase 2 (COX-2)

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Summary

Two cyclooxygenase isozymes catalyze conversion of arachidonic acid to prostaglandin H_2: constitutive COX-1 and inducible COX-2. To assess the role of COX-2 in colorectal tumorigenisis, we determined the effects of COX-2 gene (*Ptgs2*) knockouts and a novel COX-2 inhibitor on $Apc^{\Delta716}$ knockout mice, a model of human familial adenomatous polyposis. A *Ptgs2* null mutation reduced the number and size of the intestinal polyps dramatically. Furthermore, treating $Apc^{\Delta716}$ mice with a novel COX-2 inhibitor reduced the polyp number more significantly than with sulindac, which inhibits both isoenzymes. These results provide direct genetic evidence that COX-2 plays a key role in tumorigenesis and indicate that COX-2-selective inhibitors can be a novel class of therapeutic agents for colorectal polyposis and cancer.

Introduction

Through the investigation of the mechanism of action of aspirin and other anti-inflammatory drugs, cyclooxygenase (COX, PGHS, or PGH; prostaglandin-endoperoxide synthase, EC 1.14.99.1) has been established as the key enzyme responsible for prostanoid production (Vane, 1971, 1994). Conversion of arachidonic acid to prostaglandin G_2 then to prostaglandin H_2, is catalyzed by two isozymes, COX-1 and COX-2. COX-1 was initially characterized, purified, and cloned from sheep seminal vesicles (Hemler and Lands, 1976; Miyamoto et al., 1976; DeWitt and Smith, 1988) and is xpressed constitutively in many but not all mammalian tissues (O'Neill and Ford-Hutchinson, 1993). In contrast, COX-2 is induced in inflammatory cells such as monocytes and macrophages upon stimulation by cytokines (Maier et al., 1990), mitogens (Lee et al., 1992), serum (O'Banion et al., 1992), and endotoxins (Xie et al., 1992). Nonsteroidal anti-inflammatory drugs (NSAIDs) inhibit the activity of both isozymes of COX, a property that accounts for their shared therapeutic and side effect profiles (Vane, 1971; Mitchell et al., 1994). Because conventional NSAIDs such as indomethacin and sulindac inhibit both COX-1 and COX-2, newer NSAIDs have been rigorously sought that selectively inhibit COX-2 (Vane, 1994). Recently, we and others constructed mouse mutants in which the gene encoding COX-2 (*Ptgs2*) was inactivated by the knockout technology (Dinchuk et al., 1995; Morham et al., 1995). The homozygous mutant mice show abnormalities in the kidney, heart and ovaries, causing reduced viability, renal dysplasia, cardiac fibrosis, and female infertility. Although many inflammatory responses appeared unaffected, susceptibility to TNFα-induced hepatocellular toxicity was markedly reduced (Dinchuk et al., 1995).

Accumulating evidence indicates that aspirin and other NSAIDs may reduce the occurrence or progression of colorectal cancer and polyps (Marnett, 1992; Rao et al., 1995). In a randomized, double-blind, placebo-controlled study of 22 patients of familial adenomatous polyposis (FAP), patients treated with sulindac demonstrated a statistically significant decrease in the number and size of polyps (Giardiello et al., 1993). To assess the role of COX-2 in colorectal polyposis and cancer, and to evaluate the potential efficacy of COX-2 inhibitors in treating patients, we determined the effects of COX-2 gene knockout and a COX-2-selective inhibitor on intestinal polyposis using $Apc^{\Delta716}$ knockout mice, a mouse model of human FAP. Mutations in the human *APC* gene have been demonstrated to play a role in FAP as well as sporadic cancers of the entire digestive tract (Boynton et al., 1992; Horii et al., 1992; Powell et al., 1992). We recently constructed a knockout mouse strain that develops numerous polyps in the intestinal tract as early as three weeks of age due to a truncation mutation in the *Apc* gene ($Apc^{\Delta716}$) (Oshima et al., 1995a). Due to the loss of the full-length wild-type *Apc* allele (Apc^{FL}) in the proliferative zone cells, i.e., loss of heterozygosity (LOH) by the second hit, these microadenomas originated from single crypts by forming abnormal outpockets into the inner (lacteal) side of the neighboring villi. This mutant mouse strain provided a useful model system for investigation of various carcinogens, and for evaluation of anticancer and chemopreventive agents. In fact, we demonstrated recently that heterocyclic amines that are generated in overcooked meat stimulate the growth of the intestinal polyps, whereas docosahexaenoic acid (DHA) reduces the number of polyps significantly when fed to the $Apc^{\Delta716}$ mice (Oshima et al., 1995b, 1996). Here we present the data that COX-2 plays a key role in tumorigenesis and indicate that COX-2-selective inhibitors can be a novel class of therapeutic drugs for polyposis and suitable chemopreventive agents for colorectal cancers.

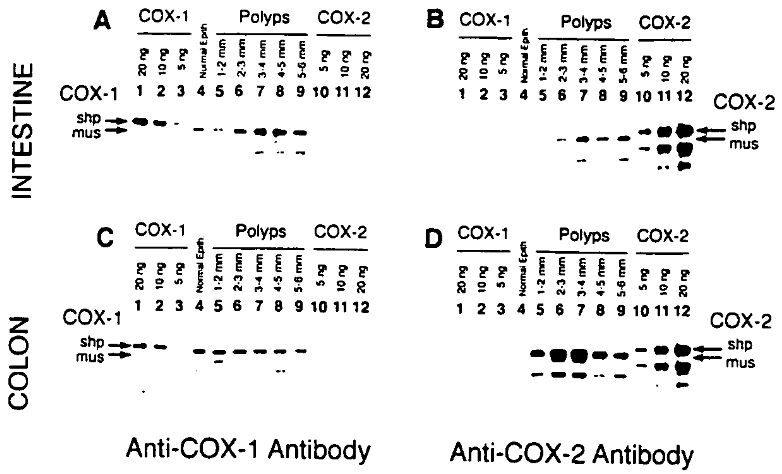

Figure 1. Immunoblot Analysis of COX-1 and COX-2 Proteins in the Normal Mouse Small Intestine and Colonic Epithelial Tissues and in Polyps Isolated from $Apc^{\Delta716}(+/-)$ Mice

(A–D) The proteins extracted from the normal epithelium (lane 4) and polyps of various sizes (lanes 5–9) were analyzed. Five, 10, and 20 ng of the purified sheep COX-1 (lanes 1–3) and COX-2 (lanes 10–12) samples were loaded as the positive controls. Samples from the small intestine (A and B) and the colon (C and D) were analyzed using polyclonal antibodies against COX-1 (A and C) or COX-2 (B and D). Note that the M_r for the sheep and mouse enzymes are different as shown by arrows on the margins (shp and mus, respectively). For the normal epithelium and polyp samples, 50 μg of microsomal protein were loaded in each lane except for lane 5 (C and D) where 30 μg of microsomal protein was loaded.

Results

Cyclooxygenase-2 (COX-2) Is Induced at Very Early Stages of Polyp Formation

To examine the expression of COX-1 and COX-2 in the $Apc^{\Delta716}$ mice, we performed immunoblot analyses of polyp proteins using specific antibodies against COX-1 and COX-2, respectively. As shown in Figure 1A, the normal intestinal epithelium, as well as the polyps of various sizes isolated from the small intestine, expressed COX-1 protein. Essentially the same result was obtained with the normal colonic epithelium and the colonic polyps (Figure 1C). These results are consistent with earlier reports that COX-1 is a constitutive enzyme (O'Neill and Ford-Hutchinson, 1993). In contrast, the normal epithelium of neither the small intestine nor the colon contained any COX-2 protein detectable by this assay (lane 4 in Figures 1B and 1D, respectively). However, small intestinal polyps larger than 2 mm in diameter and all colonic polyps (1–6 mm) contained significant levels of COX-2 protein (lanes 6–9 in Figure 1B and lanes 5–9 in Figure 1D, respectively). Small intestinal polyps smaller than 2 mm did not show a detectable level of COX-2 (lane 5 in Figure 1B). These results indicate that COX-2 is not present in the normal epithelia, but induced in the polyp tissues at a very early stage of polyp development.

COX-2 Gene (*Ptgs2*) Knockout Mutation Introduced in $Apc^{\Delta716}$ Mice Reduces the Polyp Number in a Gene Dosage-Dependent Manner

To determine the effect of the absence of COX-2 on $Apc^{\Delta716}$ polyp formation, we introduced a knockout mutation (Dinchuk et al., 1995) of the COX-2 gene (*Ptgs2*) into the $Apc^{\Delta716}$ knockout mice by two successive crosses and g nerated double-mutant mice that carried $Apc^{\Delta716}(+/-)$ *Ptgs2*$(+/-)$ and $Apc^{\Delta716}(+/-)$ *Ptgs2*$(-/-)$ mutations, respectively. The $Apc^{\Delta716}(+/-)$ *Ptgs2*$(+/+)$ litter mates were used as positive controls, which developed 652 ± 198 (SD) polyps at the age of 10 weeks. When we examined the intestinal tracts of the $Apc^{\Delta716}(+/-)$ *Ptgs2*$(+/-)$ mice at the same age, polyp numbers were reduced to 224 ± 123, i. ., ~34% of the control (statistical significance; $P = 0.0001$, Figure 2A). In the $Apc^{\Delta716}(+/-)$ *Ptgs2*$(-/-)$ mice, polyp numbers were even lower; 93 ± 98, i.e., ~14% of the control ($P < 0.0001$, Figure 2A). Moreover, the size of the polyps in the $Apc^{\Delta716}(+/-)$ *Ptgs2*$(-/-)$ mice were significantly smaller. As shown in Figure 2B, no polyps were found larger than 2.0 mm in diameter, and most polyps were <1.0 mm. Interestingly, not a single colonic polyp was found in the $Apc^{\Delta716}(+/-)$ *Ptgs2*$(-/-)$ mice, whereas the $Apc^{\Delta716}(+/-)$ *Ptgs2*$(+/-)$ and $Apc^{\Delta716}(+/-)$ *Ptgs2*$(+/+)$ mice had 1.5 ± 1.9 and 6.8 ± 7.2 colonic polyps, respectively. These results indicate that expression of the COX-2 gene increases the polyp number and size in a gene dosage-dependent manner in the $Apc^{\Delta716}(+/-)$ mutant mice. This is the first direct genetic evidence that COX-2 plays a key role in polyp formation.

Novel COX-2-Selective Inhibitor MF Tricyclic Suppresses Polyp Formation in $Apc^{\Delta716}$ Mice in a Dosage-Dependent Manner

In order to determine whether we could mimic the *Ptgs2* mutation by administration of pharmaceutical agents, we tested the effects of a novel COX-2-selective inhibitor MF tricyclic and a nonselective COX inhibitor, sulindac (Meade et al., 1993). As shown in Table 1, MF tricyclic inhibited human COX-2 but not human COX-1 stably expressed in CHO cells. In contrast, sulindac sulfide, the active form of sulindac is a potent inhibitor of both human COX-1 and COX-2. The control $Apc^{\Delta716}(+/-)$ mice fed with the drug-free diet for eight weeks had 424 ± 136 intestinal polyps (Figure 3). When fed at 14 and 3.5 mg/kg/day, MF tricyclic reduced the polyp numbers to 161 ± 60 ($P < 0.0001$) and 210 ± 110 ($P = 0.0037$), respectively. In contrast, the $Apc^{\Delta716}(+/-)$ mice fed with 12 mg/kg/day sulindac contained 312 ± 117 polyps ($P = 0.0982$; statistically not significant). Thus, MF tricyclic reduced the polyp numbers by 62% and 50% of the control at 14 mg/kg and 3.5 mg/kg, respectively, compared with only a 26% reduction of polyp number by sulindac (Figure 3). Colonic polyps in the control were 1.8 ± 1.4 whereas those in $Apc^{\Delta716}(+/-)$ mice fed 14 mg/kg and 3.5 mg/kg MF tricyclic were 0.3 ± 0.6 and 0.4 ± 0.7, respectively. The number of the colonic polyps

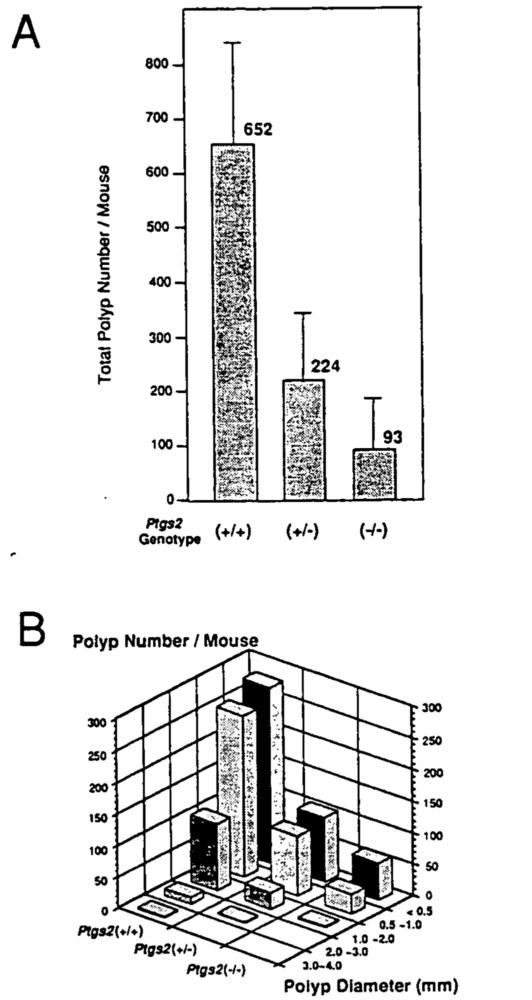

Figure 2. Effects of *Ptgs2* Mutations on Intestinal Polyps in $Apc^{\Delta716}(+/-)$ *Ptgs2* (+/−) and $Apc^{\Delta716}(+/-)$ *Ptgs2*(−/−) Mice, Compared with the $Apc^{\Delta716}(+/-)$ *Ptgs2*(+/+) Controls

(A) The mean numbers of polyps per mouse are shown with SD. (B) Size distribution of the intestinal polyps. Polyp sizes were classified according to their diameters in millimeters. Sample, n = 8 randomized mice for each group.

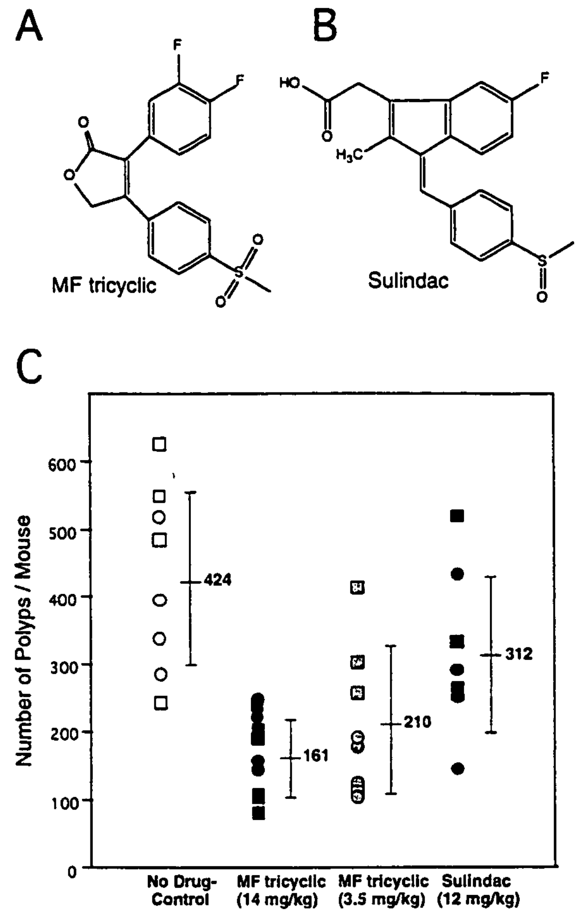

Figure 3. Effect of a Novel COX-2 Inhibitor MF Tricyclic and Sulindac on $Apc^{\Delta716}(+/-)$ Mouse Intestinal Polyps

(A) Structure of MF tricyclic.

(B) Structure of sulindac.

(C) Number of polyps per mouse scored in $Apc^{\Delta716}(+/-)$ mice fed with the control diet, or diet with MF tricyclic or sulindac.

Circles indicate polyp numbers for individual females whereas squares indicate males. The number and vertical bar to the right of each sample group indicate the mean polyp number and SD, respectively. The drug doses have been calculated from the concentrations of the drugs in the diet and the actual diet intakes.

in the mice fed 12 mg/kg sulindac were 0.9 ± 0.9. At these drug concentrations, neither MF tricyclic nor sulindac significantly affected body weights, food intake, or the general condition of the $Apc^{\Delta716}(+/-)$ mice (data not shown). The plasma concentration of MF tricyclic in the mice fed 3.5 mg/kg/day was below the detection limit of 0.1 μg/ml, whereas that in the mice fed 14 mg/kg/day was 0.45 ± 0.24 (SD) μg/ml. The mean plasma concentration of the prodrug sulindac (fed 12 mg/kg/day) was 0.54 ± 0.52 μg/ml, whereas that of sulindac sulfide, the active form of sulindac, was 0.33 ± 0.20 μg/ml and that of the inactive metabolite sulindac sulfone was 7.9 ± 4.8 μg/ml.

Table 1. COX Inhibition by MF Tricyclic and Sulindac Sulfide

	IC 50 (nM)		COX-2/COX-1
Drug	COX-1	COX-2	Ratio
MF tricyclic	>50,000	16.4	<0.0003
Sulindac sulfide	47.9	1.2	0.03

The data are the mean of 2–18 individual determinations each performed in duplicate in recombinant human COX-1 and COX-2 stably expressed in CHO cells.

Inhibition of COX-2 Causes an Alteration in Polyp Morphology

Suppression of the COX-2 activity, either by introduction of the knockout mutation or by COX-2-selective inhibitor MF-tricyclic, had a profound effect on th polyp morphology as w ll. As shown in Figure 4A, well-developed polyps (i. ., larger than the nascent ones) in $Apc^{\Delta716}(+/-)$ *Ptgs2*(−/−) mouse intestines appeared recessed from th surfac of the surrounding villi under a dissection microscope, whereas the polyps in $Apc^{\Delta716}(+/-)$ *Ptgs2*(+/+) had similar heights to the villi (Figure 4B). Histologically,

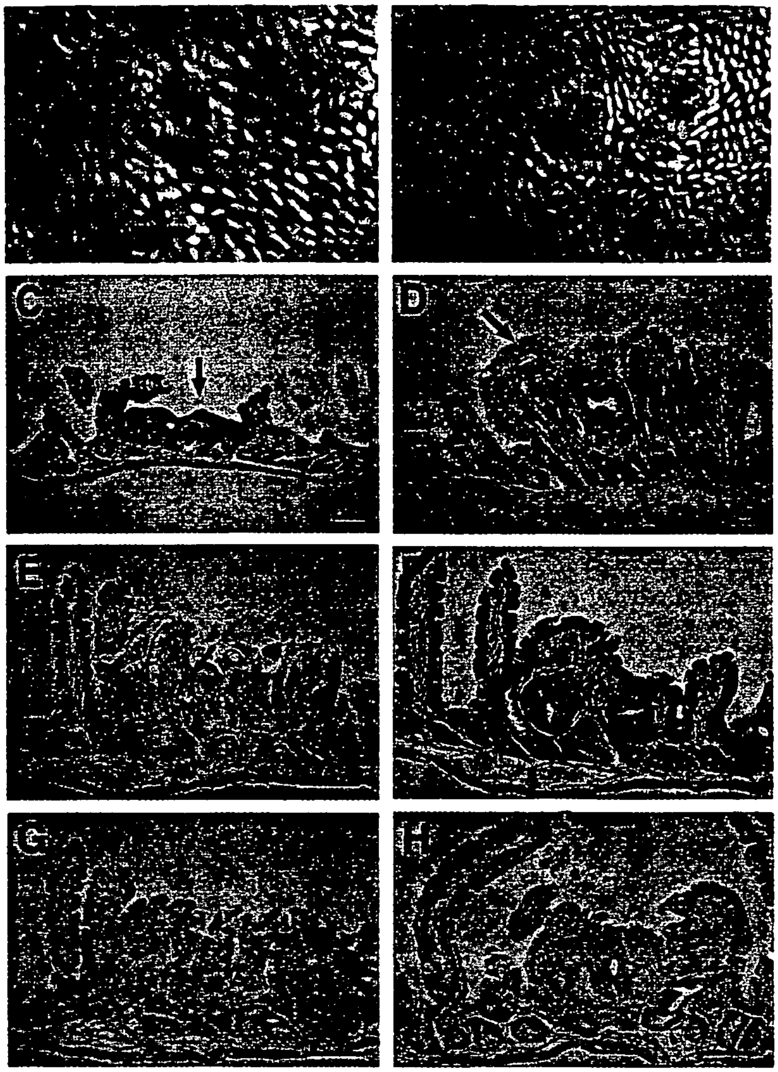

Figure 4. Polyp Morphology and Expression of COX-2 in the $Apc^{\Delta716}$ Knockout Mouse Intestine

(A and B) Dissecting micrographs of the intestinal mucosa containing well-developed polyps in $Apc^{\Delta716}(+/-)$ *Ptgs2*$(-/-)$ (A), or $Apc^{\Delta716}(+/-)$ *Ptgs2*$(+/+)$ (B) mice. Arrowheads indicate polyps viewed from the luminal side of the intestine. Scale bars, 0.5 mm.
(C and D) Histological sections of the intestinal mucosa containing well-developed polyps in $Apc^{\Delta716}(+/-)$ *Ptgs2*$(-/-)$ (C), or $Apc^{\Delta716}(+/-)$ *Ptgs2*$(+/+)$ (D) mice. Arrows indicate polyps in longitudinal sections. Hematoxilin and eosin staining. Scale bars, 100 μm.
(E) Immunohistochemical staining of LacZ protein expressed from the *Ptgs2* promoter in an intestinal polyp developed in the $Apc^{\Delta716}(+/-)$ $Ptgs2^{lacZ}(+/-)$ mouse. Arrows indicate the major sites of expression.
(F and G) Histological sections adjoining the one in (E) stained either with hematoxilin and eosin (F) or non-immune rabbit IgG for the primary antibody as a negative control (G).
(H) Immunohistochemical staining of LacZ protein in an intestinal polyp developed in the $Apc^{\Delta716}(+/-)$ *Ptgs2*$(+/+)$ mouse as a negative control for (E).
Scale bars for (E)–(G), 100 μm.

this difference is clearly shown in Figures 4C and 4D. The *Ptgs2*$(-/-)$ polyps were much flatter (shorter) than those found in *Ptgs2*$(+/+)$. Moreover, the *Ptgs2*$(-/-)$ polyps were not covered with the normal intestinal epithelium as in the *Ptgs2*$(+/+)$ polyps. Although to a lesser extent, the same tendency was observed in polyps of the $Apc^{\Delta716}(+/-)$ *Ptgs2*$(+/-)$ mice as well as of the $Apc^{\Delta716}(+/-)$ *Ptgs2*$(+/+)$ mice treated with MF-tricyclic (data not shown).

In the $Apc^{\Delta716}$ Polyps, COX-2 Is Expressed Essentially in the Interstitial Cells Rather Than in the Intestinal Epithelium

To determine the site of COX-2 expression in the $Apc^{\Delta716}$ polyps, we performed immunohistochemical analysis using the specific antibodies against COX-2. However, our antibodies were not strong enough to detect any signals in situ by immunohistochemistry (data not shown). To overcome this technical problem, we constructed another strain of *Ptgs2* knockout mouse in which one of the *Ptgs2* alleles was interrupted by a bacterial β-galactosidase gene (*lacZ*). In this construct, the *lacZ* gene was placed under the control of the *Ptgs2* promoter and expressed as a fusion protein (Figure 5). This knockout mutation was introduced into the $Apc^{\Delta716}$ knockout mice [$Apc^{\Delta716}(+/-)$ $Ptgs2^{lacZ}(+/-)$], and their polyps were analyzed. As seen in Figure 5C, the level of the COX-2 protein was reduced to a half level in the $(+/-)$ polyp tissue, and a strong band immunoreactive with the LacZ antibodies was detected. When the polyp sections were examined immunohistochemically using specific antibodies against the bacterial enzyme, a marked expression of LacZ protein was detected in the polyps (Figure 4E). Interestingly, however, its expression was found essentially in the interstitial cells with larg , ovoid and lightly stained nuclei, but not in the polyp epithelium itself (se an adjoining s ction stained by H&E; Figure 4F). The specificity of this assay was verified by two negative controls: an adjoining section stained with a non-immune rabbit IgG sample (Figure 4G) and a section of an $Apc^{\Delta716}(+/-)$ polyp stained with th LacZ antibodies, which did not detect any signals (Figure 4H).

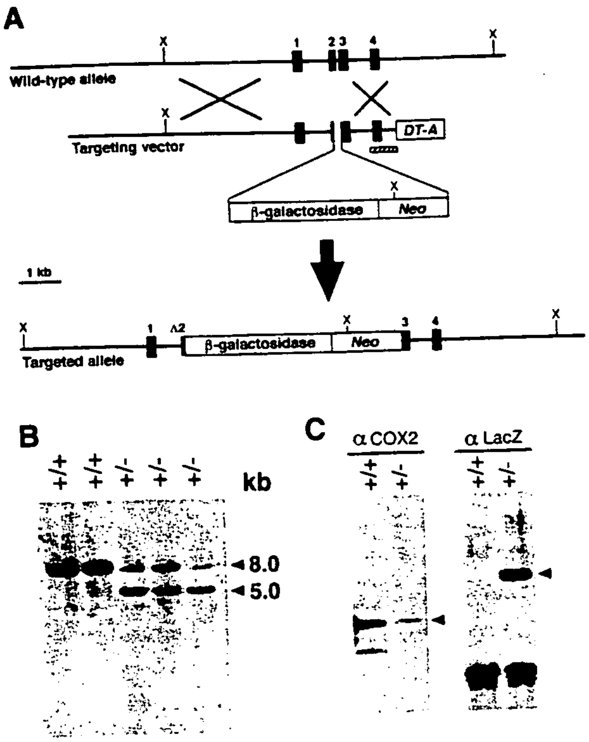

Figure 5. Construction of the $Ptgs2^{lacZ}(+/-)$ Knockout Mouse Strain by Homologous Recombination

(A) Targeting strategy showing the structures of the wild-type *Ptgs2* gene (top), the targeting vector (middle) and the targeted allele (bottom). Filled rectangles indicate exons whereas the open boxes show the bacterial β-galactosidase gene–neomycin resistance gene cassette and the diphtheria toxin α subunit selection cassette, respectively. The hatched box indicates the probe used for Southern hybridization analysis in (B). Only relevant XbaI sites are shown as X.
(B) Southern blot confirmation of the homologous recombinant ES cell clones. Genomic DNA from three recombinant ES cell clones shown as (+/−) on the top of the gel are compared with two parental ES cell samples (+/+). DNA preps were digested with XbaI and hybridized using the probe shown in (A). Note that the 8.0 kb band derived from the wild-type *Ptgs2* alleles are reduced to a half density in the (+/−) clones and a new band of 5.0 kb appeared corresponding to the knockout allele.
(C) Western immunoblot analysis of COX-2 (αCOX2, left) and LacZ (αLacZ, right) proteins in the polyp tissues developed in $Apc^{\Delta716}(+/-)$ $Ptgs2^{lacZ}(+/-)$ mice (shown as +/−) compared with polyps in the control $Apc^{\Delta716}(+/-)$ *Ptgs2*(+/+) mice (shown as +/+). Arrowheads indicate the bands for COX-2 (left) and LacZ (right). Note the COX-2 band in the (+/−) lane is of a half density as in (+/+).

Discussion

Based on epidemiological data that showed a marked reduction in mortality from colorectal carcinoma in individuals taking NSAIDs, their efficacy in reducing the number and size of the colonic polyps in human FAP has been established recently in controlled trials (e.g., Giardiello et al., 1993). Likewise, NSAIDs have been demonstrated to inhibit colorectal carcinogenesis in animal models as well (e.g., Reddy et al., 1993; Boolbol et al., 1996). Although the molecular mechanism in which NSAIDs reduce colorectal neoplasms remains unknown, a likely possibility is through their inhibition of COX. In fact, accumulating evidence suggests that colorectal carcinoma tissues from both human patients and rodent models contain elevated levels of COX-2, the inducible isozyme of COX (Eberhart et al., 1994; Kargman et al., 1995; DuBois et al., 1996; Williams et al., 1996). Here, we have demonstrated that COX-2 is induced in small polyps in the $Apc^{\Delta716}(+/-)$ intestines and colons (Figure 1). These results indicate that induction of COX-2 is a very early event in the sequence of polyp formation to colon carcinogenesis and suggest that COX-2 plays a significant role in polyp development itself. Interestingly, however, the COX-2 expression in the polyp tissues was found essentially in the interstitial cells, rather than in the polyp epithelium itself (Figure 4E). Because the major prostanoid found in the colorectal cancer tissues appear to be prostaglandin E_2 (PGE_2; e.g., Rigas et al., 1993), it is possible that the major end product of the PGH_2 generated by COX-2 induced in the interstitial cells are PGE_2. It is also conceivable that the polyp epithelium itself generates some prostacyclin (PGI_2) because it has been demonstrated in culture that rat intestinal epithelium (RIE) cells stimulated by TPA or TGFα secrete PGI_2 (DuBois et al., 1994). Tsujii and DuBois (1995) recently expressed the COX-2 gene in RIE cells at high levels. Such cells showed an increased tumorigenic potential that was reversed by sulindac. Moreover, these cells also have 3-fold increase in the duration of G_1 that may relate to the resistance of these cells to undergo apoptosis (DuBois et al., 1996). It remains to be investigated further how the expression of COX-2 in the polyp interstitial cells influences these changes and how th y stimulate tumorigenesis.

In the experiments shown in Figures 2 and 3, the number of polyps in the control $Apc^{\Delta716}$ (+/−) mice are larger than those we reported earlier (Oshima et al., 1995a). This is likely due to two reasons: improvement in polyp counting (see Experimental Procedures) and some background genes brought into $Apc^{\Delta716}$ mice from the *Ptgs2*(+/−) mice. Two genes have been reported to affect the polyp number in the Apc^{Min} mice that contain a chemically induced truncation mutation in the *Apc* gene at codon 850 (Moser et al., 1990; Su et al., 1992). When C57BL/6 Apc^{Min} mice were crossed with 129/Sv $Dnmt^{S/+}$ heterozygous mice that contained the DNA methyltransferase gene null mutation, the polyp number decreased to 41% and further to 2% when combined with a treatment with 5-azadeoxycytidine, an inhibitor of DNA methyltransferase (Laird et al., 1995). Homozygous knockout mutants of the DNA methyltransferase gene ($Dnmt^{s/s}$) are embryonic lethal (Li et al., 1992). On the other hand, a major modifier gene affecting Apc^{Min}-induced polyposis (Dietrich et al., 1993) has been proposed to encode secretory type II phospholipase A2 (gene symbol, *Pla2s*), and mouse strains that develop many polyps such as C57BL/6 and 129/Sv have greatly reduced levels of the enzyme (MacPhee et al., 1995). It is interesting that *Pla2s* encodes one of several enzymes that catalyze generation of arachidonic acid, the rate-limiting precursor of prostaglandins and leukotrienes. Because expression of *Pla2s* (e.g., in the AKR strain) reduces the polyp number rather than increases it, *Pla2s* may affect polyposis in an indirect manner such as

through changing lipid homeostasis. Accordingly, the clinical relevance of these enzymes to intestinal polyposis remains to be investigated.

Taken together, we have presented the first evidenc that COX-2 plays a key role in polyp formation and demonstrated the basis for chemopreventive treatment of polyposis and cancer by inhibitors of COX-2 such as MF tricyclic.

Experimental Procedures

Apc$^{\Delta716}$ and *Ptgs2* Knockout Mice

Constructions of *Apc*$^{\Delta716}$(+/−) mice (Oshima et al., 1995a) and *Ptgs2*(+/−) mice (Dinchuk et al., 1995) have been described recently. *Ptgs2*^{lacZ}(+/−) mice were constructed according to the strategy shown in Figure 5 as described before (Oshima et al., 1995a). Thirty-six G418-resistant ES cell clones were screened for homologous recombinant candidates by PCR with primers F (5′-CTA AAG CGC ATG CTC CAG ACT-3′, a sequence in the PGK promoter) and R (5′-ATC ACT TAG AAG CAC CGT CTC-3′, a sequence in intron 4 of *Ptgs2*). Six homologous recombinant clones were identified by Southern analysis using DIG Luminescent Detection Kit (Boehringer Mannheim). Two of them were injected into C57BL/6 blastocysts, and both were transmitted to the germline.

Apc$^{\Delta716}$(+/−) *Ptgs2*(+/−) and *Apc*$^{\Delta716}$(+/−) *Ptgs2*(−/−) Mice

Apc$^{\Delta716}$(+/−) males were mated with *Ptgs2*(+/−) females to construct *Apc*$^{\Delta716}$(+/−) *Ptgs2*(+/−) mice. Such males were crossed again with *Ptgs2*(+/−) females to generate *Apc*$^{\Delta716}$ (+/−) *Ptgs2*(−/−) mice. Offspring were genotyped by PCR as described previously (Dinchuk et al., 1995; Oshima et al., 1995a).

Polyp Number Scoring

At the scheduled age, polyps were counted according to the method described recently (Oshima et al., 1995a) except that the gut was filled with 10% formaldehyde in PBS from the anal end with the other end tied up with a thread, before opened longitudinally. Adding this procedure made the gut epithelium well distended, and the counting of the nascent uni-villous polyps was much easier and thorough.

Feeding Experiments with MF-Tricyclic and Sulindac

The *Apc*$^{\Delta716}$(+/−) mice were prepared as described recently (Oshima et al., 1995a). Eight mice (4 females and 4 males, randomized from 2–3 litters) were used for each group. After weaning at the end of the third week, mice were fed ad libitum with the diet either with or without the drug for eight weeks till the end of the 11th week. Food intakes and body weights were monitored every week and the actual drug doses were calculated accordingly. Polyps were counted as described above.

Immunoblot Quantitation of COX-1 and COX-2, and LacZ Proteins

Preparation of polyclonal antibodies against sheep COX-1 and COX-2 proteins, as well as the immunoblot analysis of human colon samples using these antibodies have been described earlier (Kargman et al., 1995). Antibodies against LacZ used for immunoblot analysis were the same as those used for immunohistochemistry below (Cappel).

Histological Analysis and Immunohistochemistry of COX-2 and LacZ Proteins

Intestinal samples were fixed overnight in 10% formaldehyde–PBS, embedded in paraffin, and sectioned at 5 μm thickness. For immunohistochemical analyses, sections were treated with 3% H_2O_2 for 1 hr to inactivate the endogenous peroxidase and incubated with 10% goat non-immune serum-3% BSA in PBS at 37°C for 60 min to block nonspecific binding. The specimens were then incubated with the primary antibodies (0.1 mg/ml anti-β-galactosidase rabbit IgG, or non-immune rabbit IgG; both from Cappel) for 60 min at room temp, and with the secondary antibodies (biotinylated goat anti-rabbit IgG; Vector Res.) followed by incubation with avidin-biotin-peroxidase complex (Vector Res.), labeled with peroxidase and colored with diaminobenzidine substrate.

Determination of COX-1 and COX-2 Catalytic Activities

Assays were performed in duplicate with recombinant human COX-1 and COX-2, respectively, stably expressed in CHO cells as described (Kargman et al., 1996).

MF Tricyclic, Sulindac, and Sulindac Sulfide

These compounds were obtained from the COX-2 project team, Merck Frosst.

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Note Added in Proof

MF tricyclic is a research compound, (3-(3,4-difluoro-phenyl)-4-(4-)methylsulfonyl)phenyl)-2-(5*H*)-furanone (Figure 3A).